Applicants : Lars Eric Sundstrom et al.
Appln. No. : 10/719,599
Page : 2

In the Specification:

Please replace the paragraph beginning at page 29, line 12 with the following amended paragraph:

- -Cultures were prepared according to the method of Stoppini *et al* (1991 J. Neurosci. Meth. 37 173-182) from 8-10 day old Wistar rat pups (Bioresources Unit, University of Southampton). Cultures were maintained *in vitro* for 14 days (37°C, 5% CO_2) during which the medium (50% minimum essential medium (MEM), 25 % Hank's balanced salt solution (HBSS), 25% heat-inactivated horse serum, supplemented with 1 mM glutamine, 5 mg/ml glucose and 1.5% fungizone) was changed every 3 days. Hypoxia was induced by replacing culture medium with serum-free (SF) medium (75% MEM, 25% HBSS, 1 mM glutamine, 5 mg/ml glucose, 1.5% fungizone) saturated with 95% N_2/5%CO_2 (and thus oxygen-free), and placing cultures in an air-tight chamber in which the atmosphere was also saturated with N_2/CO_2. After 180 minutes hypoxia, cultures were replated in normoxic SF medium and replaced in the incubator for 24 hours. Compounds were added to cultures either pre-, during and post-hypoxia (herein abbreviated to "pdp") or just in the post-hypoxic recovery period ("post") [Johnson, T. D. (1996 Trends Pharmacol. Sci. 22-27)]. Cell damage was evaluated using the fluorescent exclusion dye propidium iodide (PI, 5 μg/ml) which is normally excluded from healthy cells, but enters cells with damaged plasma membranes and becomes highly fluorescent when bound to DNA. Neuronal cell damage was quantified using the "NIH Image 1.55" software ~~(written by Wayne Rasband at the US National Institutes for Health and available from the internet by anonymous ftp from zippy.nimh.nih.gov)~~. Briefly, the area of the CA1, CA3 and dentate gyrus (DG) cell layers was measured from a transmission image. Twenty-four [[24]] hours after the commencement of hypoxia, a fluorescence image was captured using a standard Leica inverted fluorescence microscope fitted with a rhodamine filter set. The area of PI fluorescence above background in the neuronal cell layers was determined using the density slice function of Image. Cellular damage is expressed as the percentage area of the cell body layers in which PI fluorescence was detectable. After imaging, cultures were fixed overnight in 4% paraformaldehyde and stained with thionin.- -

Please replace the paragraph beginning at page 32, line 3 with the following amended paragraph:

- -After 14 days *in vitro*, organotypic hippocampal slice cultures retained much of the structure and morphology of the *in vivo* hippocampus. Specifically, clearly identifiable pyramidal (CA1 and CA3/4) and dentate gyrus granule cell layers were visible in thionin stained sections. Neurons appeared healthy, with large, lightly-stained nuclei surrounded by intensely-staining cytoplasm ~~(Figure 1)~~.- -

Please replace the paragraph beginning at page 33, line 4 with the following amended paragraph:

- -When the addition of the L-ArgSp was delayed until immediately post-hypoxia, a significant neuroprotective effect was still observed. This was concentration dependent (0.3-300 μM), with the EC_{50} lying between 3 and 30 μM. The damage observed in the CA1 subfield in these cultures was reduced (see ~~Figure~~ [[1,]] Table 1 – shown below) demonstrating that delaying the addition of the compound did not significantly reduce the neuroprotective efficacy.- -

Please replace the paragraph beginning at page 36, line 6 with the following amended paragraph:

- - ~~(Figure 2 presents an histogram demonstrating the concentration dependent neuroprotective effect of L-ArgSp (0.3-300 μM) when added immediately post-hypoxia. Neuronal damage is expressed as the percentage of the area of CA1 in which PI fluorescence was measured 24 hours following three hours of hypoxia (% Damage-CAT). ([[***]]$p<0.001$[[, **]]$p<0.01$[[, *]]$p<0.5$ vs control hypoxia (control)~~ n=108 control. n=140.3 μM, n=73 μM, n=1430 μM, n=16300 μM).- -

Please replace the paragraph beginning at page 36, line 13 with the following amended paragraph:

- -[[24]] Twenty-four hours after 180 minutes exposure to 10 μM NMDA, PI fluorescence was detectable in the CA1 subfield of the pyramidal cell layer, but not other areas

of the cultures. Increasing the concentration of NMDA to 30 μM produced a more severe insult, with significant neuronal damage occurring in both the CA1 and CA3 regions of the pyramidal cell layer, but with sparing of the granule cells of the dentate gyrus. Addition of 300 μM L-ArgSp to the medium throughout the experiment did not reduce the damage produced by either 10 μM or 30 μM NMDA. ~~Figure 3 shows a histogram demonstrating the lack of neuroprotective efficacy of L-ArgSp against NMDA mediated neurotoxicity when added post-NMDA~~. Neuronal damage is expressed as the percentage area of either CA1 (solid bars) or CA3 (hatched bars) in which PI fluorescence was measured 24 hours after 180 minutes exposure to NMDA. (mean ± sem, n=8 for each group). - -

Please replace the paragraph beginning at page 37, line 15 with the following amended paragraph:

- - Fifteen minutes global forebrain ishaemia is a particularly severe insult, producing neuronal damage throughout the hippocampal formation. When assessed 24 hours after ischaemia, animals which received vehicle alone showed a neuronal loss in CA1, CA3 and the dentate gyrus with severity being regionally dependent (CA1 > CA3 > DG). In animals treated with 1 mg/kg L-ArgSp 15 minutes prior to induction of ischaemia, the neuronal loss was significantly attenuated, particularly in the extremely vulnerable CA1 region. ~~This data is described in Figure 4 which presents a histogram demonstrating the percentage of live neurons (as determined histologically) (% Live Neurones) in CA1, CA3 and the dentate gyrus (DG) of both vehicle treated (solid bars) and L-ArgSp treated animals (hatched bars)~~.